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Art. VII.—*Further Studies in Contagious Bovine Pleuro-pneumonia Experiments to demonstrate the occurrence of two distinct types of the virus in Victoria.*

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The agglutination reaction for the diagnosis of contagious pleuro-pneumonia of bovines has been employed extensively in Victoria during the past two years with very satisfactory results. The technique employed has been fully described in a previous article (1) published by the present author.

During 1922 a number of blood samples were received for test purposes from a farm near Melbourne, on which an outbreak of disease had occurred in a herd of dairy cows, which disease was suspected to be contagious pleuro-pneumonia. The sick animals supplying the blood samples showed very high temperatures—up to 106.2° F., and also showed such clinical signs of contagious pleuro-pneumonia as coughing, rapid emaciation, and areas of dullness in the lungs.

When blood samples from these sick cows were submitted to the agglutination test (using a stock culture—referred to subsequently as "Culture Y"—as antigen) a negative reaction was obtained in each case, but on submitting the animals supplying the test sera to post-mortem examination, it was found that they, without exception, were affected with contagious pleuro-pneumonia in a very active and acute form.

From one of these sick animals a culture of the organism of contagious pleuro-pneumonia was obtained (this culture is referred to subsequently as "culture X")—which, after sub-culture in Martin's broth ox serum media for 10 generations, was agglutinated by the sera in high dilutions from sick animals from the same farm. The same sera from the same sick animals, however, had little or no agglutinating effect, even in the lowest dilutions, upon "culture Y," which was obtained from another farm many miles distant, and which had been used almost exclusively as antigen in previous agglutination tests with entirely satisfactory results.

The two cultures—"culture Y" and "culture X"—were alike in appearance and in their cultural characteristics in Martin's broth ox serum media. The fermentation reactions of both cultures were found to be identical; that is, they both gave the following reactions:—

	Saccharose	Glucose	Maltose	Lactose	Mannite	Dulcite
Acid	—	++	+	—	—	—
Gas	—	—	—	—	—	—

+ = acid; ++ = strongly acid; — = no reaction.

Both cultures, however, showed marked differences in their behaviour towards known positive sera when used as antigen for the tests of these sera. These differences can be summarised as follows:

"Culture X" plus several known positive sera gave some positive and some negative reactions.

"Culture Y" plus the same known positive sera gave positive reactions with those sera reacting negatively with "culture X," and gave negative reactions with those sera reacting positively with "culture X."

It is not proposed in this paper to enumerate all the initial experiments which have been conducted with "culture X" and "culture Y" and with known positive sera; suffice it to say that after numerous experiments and using over 30 different known positive sera, it became apparent that there were at least two distinct strains or types of the organism of contagious pleuro-pneumonia present in Victoria. These strains are only agglutinated in the higher dilutions by known positive sera of homologous type; that is to say, "culture X" is only agglutinated in high dilutions by sera from sick animals in which the causal organism is also type X, and "culture Y" is similarly only agglutinated in high dilutions by sera from sick animals in which the causal organism is type Y. Type X culture with a type Y serum invariably gives a negative reaction, as also does type Y culture with a type X serum. There is apparently no intermediate type between the two types of organism already referred to, as, in the experiments conducted, it has been found that no known positive serum has given a positive agglutination reaction to both "culture X" and "culture Y." Such a serum has always reacted positively to the one type, and negatively to the other. Typical examples of these reactions are set out in detail in Table I.

As it was apparent from the initial experiments conducted with "culture X" and "culture Y" that these cultures were only agglutinated in high dilutions by a positive serum of homologous type, it was decided to carry out a series of absorption experiments and note the behaviour of both cultures after absorption with homologous and non-homologous sera. These experiments are set out in detail in Table I. and Table II., and are summarised in Table III. Eight distinct series of agglutination tests—lettered A to H inclusive (see Table I.)—were first set up, and after incubation at 37° C. for 48 hours, the reaction which developed in each tube was carefully noted. The content of each tube was then centrifuged and 1 c.c. of the supernatent fluid from each tube in the series was then introduced into a correspondingly numbered tube in another series—lettered A(a) to H(h) inclusive; that is to say, 1 c.c. of fluid from

tube 1 in series A was put into tube 1 of series A(a), and so on. The reactions which developed after incubation for 48 hours at 37° C. are shown in Table II. already referred to.

It will be seen, on reference to these tables, that it is possible to mix "culture Y" and a positive serum (type X), and absorb all the non-specific[1] agglutinations, but at the same time the specific agglutinins for "culture X" are retained in the supernatent fluid unimpaired, and can be demonstrated by mixing 1 c.c. of that supernatent fluid with 1 c.c. of "culture X" in a subsequent test—see series B and B(b). A similar result can be obtained when a positive serum (type Y) is submitted to absorption by "culture X," and is then mixed with "culture Y"—see series E and E(e). When, however, a known positive serum is added to a culture, homologous in type with the particular serum used, a positive reaction is developed after incubation, and both the specific and the non-specific agglutinins are removed from the supernatent fluid, and this supernatent fluid, if again added to culture, whether homologous or non-homologous, gives a negative reaction even in the lowest dilutions—see series A and A(a).

TABLE I.

Tube.	Culture.	Serum 1-10	Saline.	Dilution.	Result.	Remarks.
	c.c	c.c	c.c			
1	1	1	—	1.20	+++	Serum No. 557 (type X)
2	1	.5	.5	1.40	+++	Culture Type X
3	1	.25	.75	1.80	+++	
4	1	.2	.8	1.100	++	Series A
5	1	.15	.85	1.133	+	
6	1	—	1	—	—	
7	-	1	1	—	—	
1	1	1	—	1.20	+	Serum No. 557 (type X)
2	1	.5	.5	1.40	S	Culture Type Y
3	1	.25	.75	1.80	—	Series B
4	1	.2	.8	1.100	—	
5	1	.15	.85	1.133	—	
6	1	—	1	—	—	
7	-	1	1	—	—	
1	1	1	—	1.20	+++	Serum No. 558 (type X)
2	1	.5	.5	1.40	+++	Culture (type X)
3	1	.25	.75	1.80	++	
4	1	.2	.8	1.100	++	Series C
5	1	.15	.85	1.133	++	
6	1	—	1	—	—	
7	-	1	1	—	—	

1.—The terms "specific" and "non-specific" are used throughout this paper in the restricted sense of relation to the type of the organism or serum. For example, "culture X" is specific to the serum of an animal affected with type X organism, but is non-specific to the serum of an animal affected with type Y organism.

TABLE I. (Continued)

Tube.	Culture.	Serum 1-10	Saline.	Dilution.	Results.	Remarks.
1	1	1	—	1.20	++	Serum No. 558 (type X)
2	1	.5	.5	1.40	+	Culture (type Y)
3	1	.25	.75	1.80	S	
4	1	.2	.8	1.100	—	Series D
5	1	.15	.85	1.133	—	
6	1	—	1	—	—	
7	-	1	1	—	—	
1	1	1	—	1.20	++	Serum No. 428 (type Y)
2	1	.5	.5	1.40	+	Culture (type X)
3	1	.25	.75	1.80	S	Series E
4	1	.2	.8	1.100	—	
5	1	.15	.85	1.133	—	
6	1	—	1	—	—	
7	-	1	1	—	—	
1	1	1	—	1.20	+++	Serum No. 428 (type Y)
2	1	.5	.5	1.40	+++	Culture (type Y)
3	1	.25	.75	1.80	+++	Series F
4	1	.2	.8	1.100	+++	
5	1	.15	.85	1.133	++	
6	1	—	1	—	—	
7	-	1	1	—	—	
1	1	1	—	1.20	S	Serum No. 429 (type Y)
2	1	.5	.5	1.40	V.S.	Culture (type X)
3	1	.25	.75	1.80	—	Series G
4	1	.2	.8	1.100	—	
5	1	.15	.85	1.133	—	
6	1	—	1	—	—	
7	-	1	1	—	—	
1	1	1	—	1.20	+++	Serum No. 429 (type Y)
2	1	.5	.5	1.40	+++	Culture (type Y)
3	1	.25	.75	1.80	++	Series H
4	1	.2	.8	1.100	++	
5	1	.15	.85	1.133	++	
6	1	—	1	—	—	
7	-	1	1	—	—	

+++ = Agglutination and sedimentation of agglutinated organisms with complete naked eye clearing of the supernatent fluid.
++ = Agglutination with well-defined deposit, fluid nearly clear.
+ = Marked flocculent agglutination and some sedimentation, fluid not clear.
S = Slight agglutination deposit, fluid not clear.
— = No naked eye trace of agglutination or clearing of fluid.

TABLE II.

Tube.	Culture.	Fluid from Series A.	Saline.	Dilution.	Result.	Remarks.
1	1 c.c.	1 c.c. tube 1	—	1.40	—	Serum No. 557 (Type X)
2	1 c c	1 c.c. tube 2	—	1.80	—	Culture (Type X)
3	1 c.c.	1 c.c. tube 3	—	1.160	—	
4	1 c.c.	1 c.c. tube 4	—	1.200	—	
5	1 c.c.	1 c.c. tube 5	—	1.266	—	Series A(a)
6	1 c.c.	1 c.c. tube 6	—	—	—	
7	—	1 c.c. tube 7	1 c.c.	—	—	
		FLUID FROM B				
1	1 c.c.	1 c.c. tube 1	—	1.40	+++	Serum No. 557 (Type X)
2	1 c.c.	1 c.c. tube 2	—	1.80	+++	Culture (Type X)
3	1 c.c.	1 c.c. tube 3	—	1.160	+	
4	1 c.c.	1 c.c. tube 4	—	1.200	+	
5	1 c.c.	1 c.c. tube 5	—	1.266	+	Series B(b)
6	1 c.c.	1 c.c. tube 6	—	—	—	
7	—	1 c.c. tube 7	1 c.c.	—	—	
		FLUID FROM C				
1	1 c.c.	1 c.c. tube 1	—	1.40	? V.S.	Serum No. 558 (Type X)
2	1 c.c.	1 c.c. tube 2	—	1.80	—	Culture (Type Y)
3	1 c.c.	1 c.c. tube 3	—	1.160	—	
4	1 c.c.	1 c.c. tube 4	—	1.200	—	
5	1 c.c.	1 c.c. tube 5	—	1.266	—	Series C(c)
6	1 c.c.	1 c.c. tube 6	—	—	—	
7	—	1 c.c. tube 7	1 c.c.	—	—	
		FLUID FROM D				
1	1 c.c.	1 c.c. tube 1	—	1.40	—	Serum No. 558 (Type X)
2	1 c.c.	1 c.c. tube 2	—	1.80	—	Culture (Type Y)
3	1 c.c.	1 c.c. tube 3	—	1.160	—	Series D(d)
4	1 c.c.	1 c.c. tube 4	—	1.200	—	
5	1 c.c.	1 c.c. tube 5	—	1.266	—	
6	1 c.c.	1 c.c. tube 6	—	—	—	
7	—	1 c.c. tube 7	1 c.c.	—	—	
		FLUID FROM E				
1	1 c.c.	1 c.c. tube 1	—	1.40	+++	Serum No. 428 (Type Y)
2	1 c.c.	1 c.c. tube 2	—	1.80	+++	Culture Type (Y)
3	1 c.c.	1 c.c. tube 3	—	1.160	++	
4	1 c.c.	1 c.c. tube 4	—	1.200	++	Series E(e)
5	1 c.c.	1 c.c. tube 5	—	1.266	+	
6	1 c.c.	1 c.c. tube 6	—	—	—	
7	—	1 c.c. tube 7	1 c.c.	—	—	

Table II. (Continued)

Tube.	Culture.	Fluid from Series A.	Saline.	Dilution.	Result.	Remarks.
		FLUID FROM F				
1	1 c.c.	1 c.c. tube 1	—	1.40	?V.S.	Serum No. 428 (Type Y)
2	1 c.c.	1 c.c. tube 2	—	1.80	—	Culture (Type X)
3	1 c.c.	1 c.c. tube 3	—	1.160	—	Series F(f)
4	1 c.c.	1 c.c. tube 4	—	1.200	—	
5	1 c.c.	1 c.c. tube 5	—	1.266	—	
6	1 c.c.	1 c.c. tube 6	—	—	—	
7	—	1 c.c. tube 7	1 c.c.	—	—	
		FLUID FROM G				
1	1 c.c.	1 c.c. tube 1	—	1.40	+++	Serum No. 429 (Type Y)
2	1 c.c.	1 c.c. tube 2	—	1.80	++	Culture (Type Y)
3	1 c.c.	1 c.c. tube 3	—	1.160	++	
4	1 c.c.	1 c.c. tube 4	—	1.200	+	Series G(g)
5	1 c.c.	1 c.c. tube 5	—	1.266	S	
6	1 c.c.	1 c.c. tube 6	—	—	—	
7	—	1 c.c. tube 7	1 c.c.	—	—	
		FLUID FROM H				
1	1 c.c.	1 c.c. tube 1	—	1.40	—	Serum No. 429 (Type Y)
2	1 c.c.	1 c.c. tube 2	—	1.80	—	Culture (Type X)
3	1 c.c.	1 c.c. tube 3	—	1.160	—	Series H(h)
4	1 c.c.	1 c.c. tube 4	—	1.200	—	
5	1 c.c.	1 c.c. tube 5	—	1.266	—	
6	1 c.c.	1 c.c. tube 6	—	—	—	
7	1 c.c.	1 c.c. tube 7	1 c.c.	—	—	

TABLE III.

(Summary of Tables I. and II.).

Series A	Type X culture + Type X serum	=Positive reaction in all dilutions tested.
Series A(a)	Fluid from Series A + Type X culture	=Negative reaction, all agglutinins absorbed by the reaction developed in Series A
Series B	Type Y culture + Type X serum	=Negative reaction—only non-specific. Agglutinins give slight reaction in the two lowest dilutions.
Series B(b)	Fluid from Series B + Type X culture	=Positive reaction. Specific agglutinins not removed by the absorption experiment in Series B
Series C	Type X culture + Type X serum	=Positive reaction.
Series C(c)	Fluid from Series C + Type Y culture	=Negative reaction. Both specific and non-specific agglutinins absorbed by the experiment in series C
Series D	Type Y culture + Type X Serum	=Negative reaction. Only non-specific agglutinins reacting in lowest dilutions.
Series D(d)	Fluid from Series D + Type Y culture	=Negative reaction. All agglutinins to Type Y culture absorbed by experiment in series D
Series E	Type X culture + Type Y serum	=Negative reaction
Series E(e)	Fluid from Series E + Type Y culture	=Positive reaction. Specific agglutinins not absorbed by experiment in Series E
Series F	Type Y culture + Type Y serum	=Positive reaction.
Series F(f)	Fluid from Series F + Type X culture	=Negative reaction. Both specific and non-specific agglutinins absorbed by experiment in Series F
Series G	Type X culture + Type Y serum	=Negative reaction.
Series G(g)	Fluid from Series G + Type Y culture	=Positive reaction
Series H	Type Y culture + Type Y serum	=Positive reaction
Series H(h)	Fluid from Series H + Type X culture	=Negative reaction. Both specific and non-specific agglutinins absorbed by experiment in SeriesH

Reviewing the foregoing experiments, it is apparent that there are at least two distinct strains or types of the organism of contagious pleuro-pneumonia responsible for outbreaks of the diseases in Victoria. These two types are identical in their cultural characteristics and fermentation reactions, but differ in the manner of their behaviour when mixed with sera from animals affected with contagious pleuro-pneumonia.

The majority of outbreaks of contagious pleuro-pneumonia in Victoria appear to be produced by the type Y organism, but owing to the possibility of the other type being the causal organism in any outbreak, it has been found necessary, when conducting agglutination tests with suspected sera, to first ascertain the type of the organism responsible for the particular outbreak under investigation. Whenever possible, a known positive serum has been obtained from each outbreak to serve as a control. This known positive serum is tested with type X culture and type Y culture separately, and the extent of the reaction which is developed with each type of culture is carefully noted. In that way the type of the organism responsible for the outbreak is ascertained and sera from all suspected animals in the same herd can then be tested with a culture of the organism of the same type as that which is causing the disease in the herd.

Whenever a known positive serum is unobtainable from the outbreak in which the suspected animals are, it becomes necessary to test each suspected serum twice—firstly, against type X, and, secondly, against type Y, each separately—before it can be definitely stated that the animal supplying the sample is affected with or is free from the disease. A culture emulsion containing equal parts of types X and Y combined did not give satisfactory results, excepting in very acute cases of the disease, and was found to be very unreliable when the serum sample being tested was from an animal in which the disease was chronic.

(The agglutination reaction in contagious pleuro-pneumonia is always more marked and definite with the serum from an animal in which the disease is acute than that from an animal in which the disease is chronic.)

Prophylactic Inoculation.

Having established the fact that there are at least two distinct types of the organism of contagious pleuro-pneumonia present in Victoria, the question of prophylactic inoculation in the tail with culture or virus may have to be considered in the light of this knowledge. For instance, a prophylactic inoculation with culture or virus containing only type X organism may protect against type X organism, but not against type Y, and *vice versa*. It is worthy of mention that before the use of pure culture of the organism of contagious pleuro-pneumonia for prophylactic inoculation became so general in Victoria, experienced stock-owners have noted and commented upon the fact that the immunity obtained from tail inoculation appeared to be greatest when a sick animal was killed on the

farm, the virus collected from it, and used to inoculate all the contact animals of the herd. If "foreign" virus—that is, virus obtained on another farm, usually in another district—was used, they noted that very often there was very little, if any, immunity set up in the animals in which the "foreign" virus was inoculated. This observation, based as it was on empiric grounds, may have some foundation in the fact that when virus obtainable on the farm was used for prophylactic inoculation of the contact animals on the same farm, the type of organism present in the virus inoculated would be of the same type as that causing the disease on the farm, whereas when a "foreign" virus was used, the organism might or might not have been homologous in type with that causing the disease on the farm.

Experiments are now being conducted at the Veterinary Research Institute, Melbourne University, to ascertain whether the organism of one type will protect only against its own type, or will protect against both types.

LITERATURE.

(1) Heslop (1922): "Further researches into the serological diagnosis of contagious pleuro-pneumonia of cattle."—*Journal Comparative Pathology and Therapeutics*, vol. xxxv., part 1, March 1922, pp. 1-12.